Substrate-dependent reversal of anion transport site orientation in the human red blood cell anion-exchange protein, AE1

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The tightly coupled, one-for-one exchange of anions mediated by the human red blood cell AE1 anion-exchange protein involves a ping-pong mechanism, in which AE1 alternates between a state with the anion-binding site facing inward toward the cytoplasm (Ei) and a state with the site facing outward toward the external medium (Eo). The conformational shift (Ei ↔ Eo) is only permitted when a suitable substrate such as Cl^- or HCO_3^- (B^-) is bound. With no anions bound, or with Cl^- bound, far more AE1 molecules are in the inward-facing than the outward-facing forms (Ei ≫ Eo, ECli ≫ EClo). We have constructed a model for Cl^-–B^- exchange based on Cl^-–Cl^- and B^-–B^- exchange data, and have used it to predict the heteroexchange flux under extremely asymmetric conditions, with either all Cl^- inside and all B^- outside (Cli-Bo) or *vice versa* (Bi-Clo). The experimental values of the ratio of the exchange rate for Bi-Clo to that for Cli-Bo are only compatible with the model if the asymmetry of bicarbonate-loaded sites (A_B = EBo/EBi) > 10, the opposite of the asymmetry for unloaded or Cl-loaded sites. Furthermore, the Eo form has a higher affinity for HCO_3^- than for Cl^-, whereas the Ei form has a higher affinity for Cl^-. The fact that this ''passive'' system exhibits changes in substrate selectivity with site orientation (''sidedness''), a characteristic usually associated with energy-coupled ''active'' pumps, suggests that changes in affinity with changes in sidedness are a more general property of transport proteins than previously thought.

It has long been known that energy-coupled transporters like the Na,K-ATPase are able to transport ions against their electrochemical gradients because the affinity of the ion-binding site changes when it is translocated from one side of the membrane to the other. For example, Na^+ affinity is high for the inward-facing E_1 Na,K-ATPase form and low for the outward-facing E_2 form, whereas K^+ affinities have the opposite relationship (1). So far, no such asymmetry has been shown for ''passive'' transport systems in which ions move down their electrochemical gradients.

The AE1 anion-exchange protein in human red blood cells seems to operate by a ping-pong mechanism, in which transport of Cl^- or HCO_3^- across the membrane involves conversion of a form with the transport site facing inward (Ei) to a form with the site facing outward (Eo) or *vice versa*, a conversion that only occurs if a suitable anion is bound to the site (2–5). For both the unloaded (6, 7) and the Cl-loaded (8) forms of AE1, the free energies of the inward-facing forms are much lower than those for the outward-facing forms, so Ei ≫ Eo and ECli ≫ EClo.

The possibility that AE1 might exhibit different asymmetric properties with respect to its two physiological substrates, Cl^- and HCO_3^-, was first suggested by a brief report (9) that the rate of exchange of internal HCO_3^- (Bi) for external Cl^- (Clo), Bi-Clo exchange, was faster than the reverse (Cli-Bo) exchange rate at 38°C. This finding was attributed to a differential inhibitory effect of these ions acting at an external ''modifier'' site, which later measurements failed to confirm (10, 11). Recently, we reinterpreted these data in light of a model (12) based on more complete kinetic information on Cl^-–Cl^- (10) and HCO_3^-–HCO_3^- (13) exchange, and hypothesized that at 38°C most of the HCO_3-loaded transport sites face outward rather than inward as do the empty or Cl-loaded sites (13).

This conclusion rested on only two data points at 38°C (9), where anion exchange is very fast ($t_{1/2} \approx 50$ ms) and difficult to measure. To test the hypothetical asymmetry more rigorously, we have made far more extensive measurements of anion exchange under extremely asymmetric conditions and at 0°C where precise flux measurements are much easier to obtain.

Materials and Methods

Cell Preparation. Red blood cells were obtained from volunteers with informed consent and were washed three times in ice-cold 150 KH {150 mM KCl/24 mM sucrose/20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)], pH 6.9 at room temperature} to remove white cells and fibrin. Red cells were divided into two portions: one was washed three times at room temperature with 150 KH, pH 7.8 (at 0°C) and the other with 150 $KHCO_3$ (pH 7.8 at 0°C, like 150 KH but with $KHCO_3$ replacing KCl). Bicarbonate-loaded cells were treated with 1 mM acetazolamide at 38°C for 20 min, washed, and then treated again, a method that effectively inhibits carbonic anhydrase activity (7, 11).

Flux Measurements. Flux procedures were performed essentially as described by Gasbjerg and Brahm (7). Briefly, cells were loaded at pH 7.8 at 0°C either with ^{36}Cl or with $H^{14}CO_3$ for ≥10 min and then kept on ice. After centrifugation and removal of supernatant, the cells were resuspended in either 150 KH or 150 $KHCO_3$ medium, pH 7.8 at 0°C and then rapid samples were taken with syringe filters. A logarithmic function—$\ln[P_{inf}/(P_{inf} - P)]$ of the radioactivity of the filtrate, P, in cpm at the indicated time and normalized to the radioactivity of an equal aliquot of the cell suspension, P_{inf}—was plotted against time in seconds. A least-squares fit of these points to a straight line gives a slope that is equal to the rate constant, k, for anion exchange (7, 14). To facilitate comparison of the slopes, the value of the y-intercept of the initial least-squares best-fit line was subtracted from each point, and then the data were replotted so that all lines pass through the origin. Intracellular concentrations of Cl^- and HCO_3^- were measured as described (7). The mean values were 116 ± 2 mM (SEM, $n = 4$) for [Cli] in 150 KH medium and 112 ± 4 mM ($n = 3$) for [HCO_3i] in 150 $KHCO_3$ medium and were not significantly different ($P = 0.5$). The measured intracellular Cl^- content of cells in 150 KH was 178 ± 5 mmol/kg dry solids, which was slightly larger than the HCO_3^- content of cells in 150 $KHCO_3$ medium, 163 ± 8 mmol/kg dry solids. This difference was not significant ($P = 0.2$), but even if it is real, it only decreases the

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Abbreviations: B^-, HCO_3^-; AE1, anion exchanger 1 protein; Ei, form of AE1 with transport site facing toward cytoplasm; Eo, form with transport site facing external medium; A_{Cl}, asymmetry ratio for Cl^- (= EClo/ECli); A_B, asymmetry ratio for bicarbonate (= EBo/EBi).

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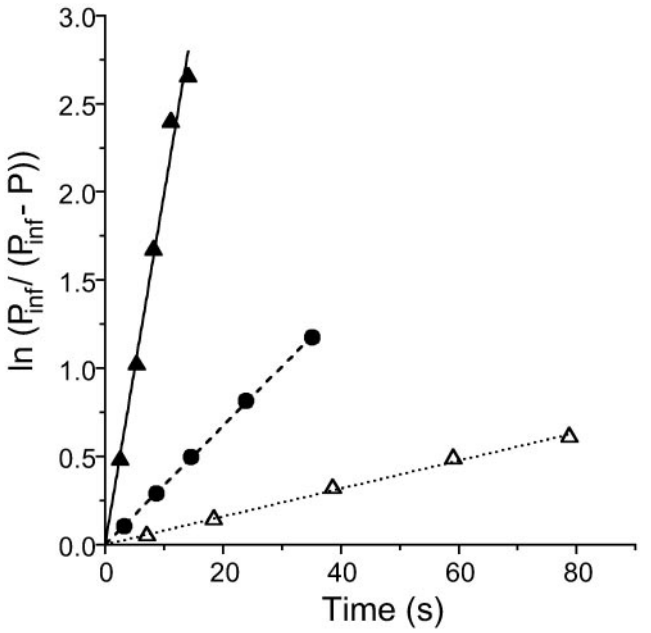

Fig. 1. Efflux of $^{36}Cl^-$ from red blood cells into 150 mM HCO_3^- medium (150 $KHCO_3$) or of $H^{14}CO_3^-$ into 150 mM Cl^- medium (150 KH). Mean values for [Cli] and for [HCO_3i] are given in *Materials and Methods* and in Fig. 2. Because the internal anion content is nearly the same in all experiments, rate constants (*k*) are proportional to the fluxes (*J*). Solid line and black triangles, Bi-Clo, $k = 0.199 \pm 0.015\ s^{-1}$; dashed line and black circles, Cli-Bo, $k = 0.0336 \pm 0.0003\ s^{-1}$; dotted line and white triangles, Bi-Clo with 10 μM DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate) added to the flux medium, $k = 0.0079 \pm 0.0003\ s^{-1}$.

ratio of Bi-Clo to Cli-Bo fluxes by 8% relative to that estimated from the exchange rate constants.

Statistical Analysis. Values are presented as mean ± SEM, based on the number of flux measurements with cells from three different donors. Ratios of exchange rate constants for Bi-Clo and Cli-Bo were determined by dividing the individual Bi-Clo exchange rate constants by the mean of at least four determinations of the Cli-Bo rate constant with blood from the same donor. *P* values are estimated from Student's *t* test for unpaired samples.

Results

Comparison of Bicarbonate-Chloride and Chloride-Bicarbonate Heteroexchange Rates. To maximize the experimentally observable effects of different distributions of Cl^- or HCO_3^--loaded sites, we measured anion heteroexchange under the most asymmetric conditions that could be attained in isotonic media; that is, with either all Cl^- inside and all HCO_3^- outside (Cli-Bo, black circles in Fig. 1) or with all HCO_3^- inside and all Cl^- outside (Bi-Clo, black triangles in Fig. 1). Note that the rate constant for efflux of HCO_3^- into Cl^- medium (given by the slope of the solid line) is much greater than the rate constant for efflux of Cl^- into HCO_3^- medium (given by the slope of the dashed line). The ratio of the rate constant for Bi-Clo exchange to that for Cli-Bo exchange in 15 measurements with three blood donors was 5.7 ± 0.3 (SEM). The white triangles and dotted line show the data for Bi-Clo conditions, but with 10 μM DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate) added to the flux medium. The fact that DIDS inhibits over 95% of the bicarbonate efflux demonstrates that the transport of $H^{14}CO_3$ across the membrane as $^{14}CO_2$ (which should not be inhibited by DIDS) is negligible under these conditions, presumably because carbonic anhydrase was inhibited by acetazolamide (7). Also, the HCO_3^- efflux rate constant was 54 to 65% higher (for different donors) with Cl^- outside than with HCO_3^- outside (data not shown), which would not be expected for CO_2 diffusion. Thus, $^{14}CO_2$ diffusion cannot account for the much larger anion exchange rate constants observed under Bi-Clo conditions than those under Cli-Bo conditions.

Table 1. Dissociation constants ($K_{1/2}$) in mM for Cl^- and HCO_3^- binding to AE1

	Calculated true $K_{1/2}$ for Ei and Eo		Apparent $K_{1/2}$ under symmetric conditions, with Xi = Xo†	
Anion	Ei*	Eo*	Calculated*	Observed ± SEM‡
Cl^-	48	20	44	50.1 ± 9.5
HCO_3^-	340	2	33	30.4 ± 3.0
Cl^-/HCO_3^-	0.14	10	1.33	1.65

*Calculated from the self-consistent ping-pong model, with A (Ei/Eo with Cli = Clo or Bi = Bo) = 0.058892, A_{Cl} = ECli/EClo = 0.14 and A_B = 10.
†Apparent half-saturation concentration with Cli = Clo or Bi = Bo.
‡From ref. 7. In addition to the modifications of the symmetrical (Xi = Xo) half-saturation concentrations shown, to obtain a self-consistent model, the observed half-saturation concentrations for external Cl^- and B^-, with internal concentration held constant (not shown), were adjusted by ≤0.11 mM [SEMs were ≥0.2 mM (7)].

Quantitative Model. To interpret the data quantitatively, we constructed a self-consistent ping-pong model for Cl^-–B^- exchange based on Cl^-–Cl^- and B^-–B^- exchange data at 0°C (7). Self-consistency is a concern, because the raw experimental data for Cl^- and B^- exchange do not give precisely the same ratio, A, of Eo to Ei with Xi = Xo (where X^- refers to any anion, such as Cl^- or B^-). This ratio is determined solely by the free-energy difference between Ei and Eo, and so must be a constant, independent of which anion substrate is used (15). Following the method of Weinstein (12), we allowed the experimentally determined parameters (half-saturation and maximum flux, with anion concentrations changed either at both sides of the membrane or only at the external side and corrected for self-inhibition; ref. 7) to vary within the limits of their standard errors until a self-consistent mathematical model was obtained (Table 1). For both Cl^- and B^-, the A values calculated from the four different determinations of external anion half-saturation (7) were averaged.

The A value for the self-consistent model was 0.058892 (the precision being required so that the model would predict zero net fluxes under conditions where this is required thermodynamically), which is not far from the value of 0.064 calculated in the paper (5) that first demonstrated this asymmetry. The model is based on the assumption that the binding of anions at the two sides of the membrane is fast compared with the rate of anion translocation across the membrane. Evidence from ^{35}Cl and ^{37}Cl NMR supports this assumption for Cl^- (16); there is no evidence on this point for HCO_3^-. The calculated apparent $K_{1/2}$ values for Cl^- and B^- under symmetric conditions (Table 1) fall within the errors of the observed values. The ratio of the $K_{1/2}$s for Cl^- and B^- (Table 1, Calculated) is somewhat smaller than that (≥2) obtained from anion substitution experiments by Gasbjerg and Brahm (figure 8 of ref. 9). The latter value is also larger than the ratio calculated from a much larger series of saturation experiments in the same paper (Table 1, Observed) and thus may reflect inaccuracies in the anion substitution method and/or some as yet undiscovered complexities of the system.

Determination of A_B. Next, we calculated the Cl^-–B^- exchange fluxes under the extremely asymmetric conditions used for the

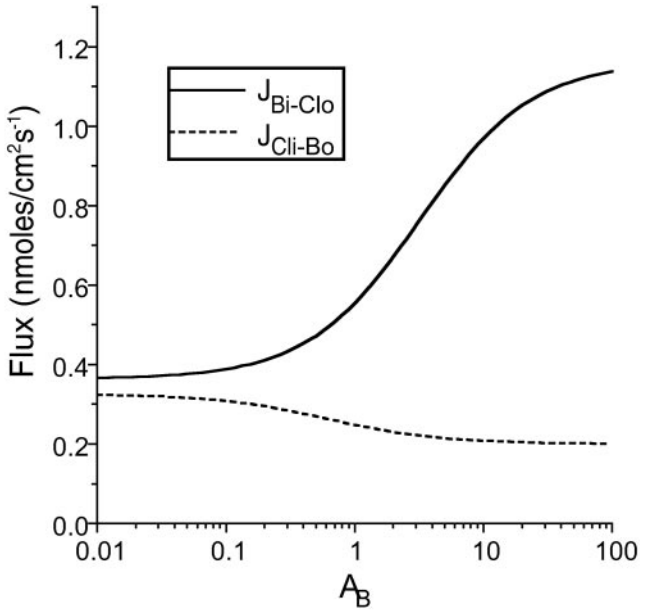

Fig. 2. Calculation of the heteroexchange fluxes with Cl^- inside and HCO_3^- outside ($J_{Cli\text{-}Bo}$, dashed line) or HCO_3^- inside and Cl^- outside ($J_{Bi\text{-}Clo}$, solid line). The fluxes were calculated (12, 13) for various values of A_B (= EBo/EBi), with the self-consistent model parameters from Table 1, so that the maximum Cli-Clo and Bi-Bo fluxes would be consistent with experimental data (7). The mean values for [Cli] (116 ± 2 mM, SEM, $n = 4$) and [HCO_3i] (112 ± 4 mM, $n = 3$) from the experiments were used, and A_{Cl} (= ECli/EClo) was 0.14 (S. D. Kennedy, C. A. Wu, and P.A.K., unpublished data; see text for details).

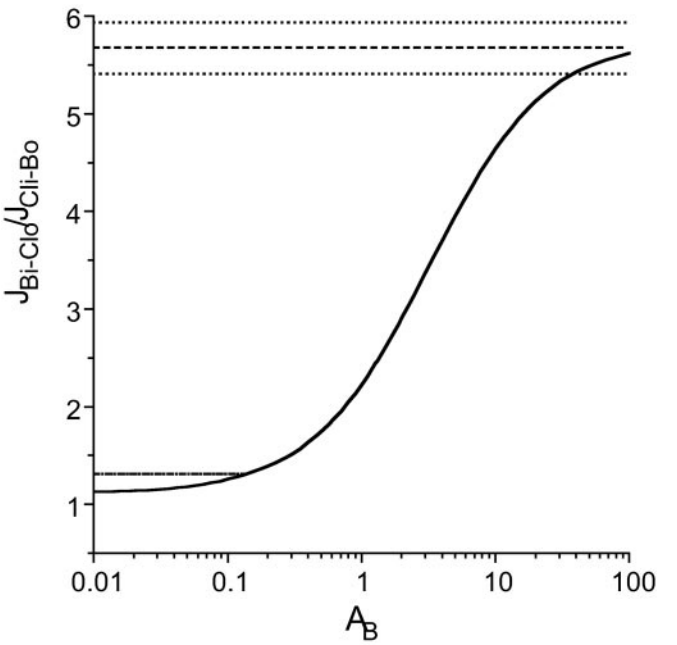

Fig. 3. Ratio of HCO_3^- efflux with Bi-Clo to Cl^- efflux with Cli-Bo (solid line). Ratios were determined as a function of A_B from calculated fluxes in Fig. 2. Dash-dot line shows value (1.3) expected for ratio if A_B is the same as A_{Cl}, 0.14. Dashed line shows mean of efflux rate constant values from experiments as in Fig. 1, with dotted lines indicating ±1 SEM.

experiments; that is, with all Cl^- inside and all B^- outside (Cli-Bo), or with all B^- inside and all Cl^- outside (Bi-Clo). The fluxes were calculated as a function of the asymmetry ratio for bicarbonate-loaded sites, A_B (= EBo/EBi). Calculations were made with A_{Cl} (= EClo/ECli) = 0.14, based on data obtained recently with an improved ^{35}Cl NMR technique (S. D. Kennedy, C. A. Wu, and P.A.K., unpublished data). This value is slightly higher than that (0.11) reported earlier (8).

Bi-Clo flux increases with increasing A_B (Fig. 2, solid line), whereas the Cli-Bo flux decreases (dashed line). The ratio of the Bi-Clo flux to the Cli-Bo flux thus strongly depends on A_B (Fig. 3), so a measurement of this ratio can give information about the bicarbonate-loaded site asymmetry. If the bicarbonate asymmetry ratio were similar to that for unloaded or Cl-loaded sites ($\ll 1$), then the flux ratio should be low, about 1.3 (see horizontal dash-dot line near the bottom of Fig. 3). The flux ratio from the measured rate constants, 5.7 ± 0.3 (dashed line, with dotted lines showing ± 1 SEM), is far higher and indicates a bicarbonate asymmetry ratio, A_B, ≥ 10, which is completely opposite to the asymmetry ratio ($\ll 1$) for unloaded or Cl-loaded sites. This result holds true even if the flux ratio is corrected to 5.2 ± 0.2 to take into account the slightly larger measured anion content for cells preequilibrated in Cl^- medium as compared with bicarbonate medium (see *Materials and Methods*) and even if the lower A_{Cl} value (0.11) reported (8) is used.

Although the heteroexchange data fit well to a model assuming exponential anion exchange kinetics (Fig. 1), the model with opposite asymmetries for Cl^- and B^- predicts that Cl–B heteroexchange kinetics should not be perfectly exponential (see Figs. 5 and 6, which are published as supporting information on the PNAS web site, www.pnas.org). With $A_B = 10$, the initial Bi-Clo flux at very short times (<2 s) is larger than that measured at the times our samples were taken, whereas the initial Cli-Bo flux is smaller. Thus, our data should underestimate the Bi-Clo flux and overestimate the Cli-Bo flux, giving an apparent A_B value somewhat lower than the true value (see Figs. 2 and 3). When the errors introduced by exponential analysis of the data are considered, therefore, the evidence showing that $A_B \gg A_{Cl}$ is strengthened, rather than weakened.

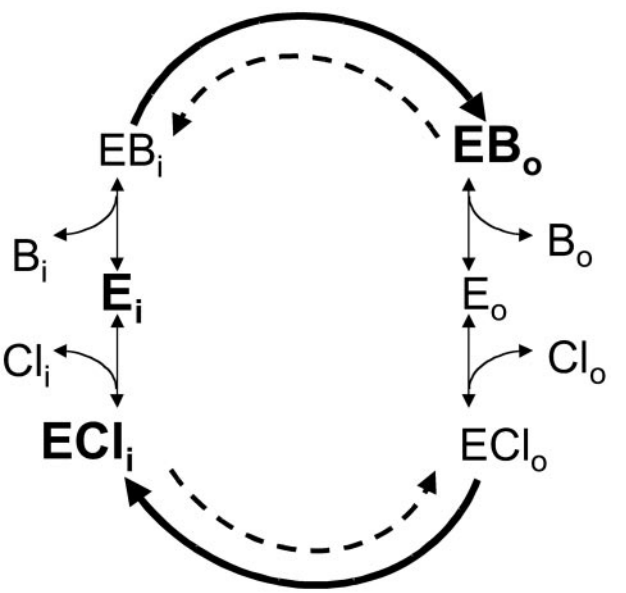

Fig. 4. Rationale for asymmetry in Cli-Bo and Bi-Clo fluxes. The majority forms of AE1 are shown in bold type: Ei ≫ Eo, ECli ≫ EClo, and EBo ≫ EBi. The asymmetries for Cl^- and HCO_3^-–loaded sites imply that the rate constants for transitions between these forms must be asymmetric, with the solid line indicating the faster rate constant in each case. For the clockwise cycle, Bi-Clo exchange, the faster steps are involved for both bicarbonate and chloride transport across the membrane (black solid lines), but for the anticlockwise cycle, Cli-Bo exchange, the slower steps are used (dashed lines).

PHYSIOLOGY

Discussion

Explanation of Asymmetry. Fig. 4 shows qualitatively the major reason for the flux asymmetry: Bi-Clo exchange (clockwise cycle, black solid lines) utilizes the ion-translocation process with the faster rate constant in each case (black solid lines), whereas Cli-Bo exchange uses the slower steps (anticlockwise cycle, dashed lines).

Cl^-/HCO_3^- Selectivity of Inward-Facing and Outward-Facing Transport Sites. Table 1 shows the calculated values for the dissociation constants for Cl^- and B^- binding to Ei and Eo based on an A_B value of 10. For binding to Eo, B^- has about a 10-fold higher affinity than Cl^-. For Ei, the B^- dissociation constant strongly depends on the exact value of A_B; for $A_B = 10$, it is over 7 times as large as the Cl^- dissociation constant. Thus, Ei has a higher affinity for Cl^- than for B^-, whereas Eo has the reverse selectivity, with a higher B^- affinity.

Physiological Effects of Asymmetry. The strongly asymmetric Cli-Bo and Bi-Clo fluxes shown in Fig. 1 are seen under extremely asymmetric conditions. With the much smaller gradients for Cl^--B^- exchange that exist in capillaries ($\approx$2 mM), the asymmetry in HCO_3^- and Cl^- affinities is predicted to have only a negligible effect on the rates of bicarbonate uptake vs. bicarbonate release (13). Because both symmetric and asymmetric models must fit the constraints imposed by measurements of anion homoexchange (7, 10–13), alterations in anion affinities are compensated by changes in the rate constants for the anion translocation steps so as to yield nearly the same net anion fluxes under physiological conditions (see *Appendix*, which is published as supporting information on the PNAS web site). Under the conditions in the kidney outer medullary collecting duct (12), where a truncated variant of AE1 is expressed (17), the highly asymmetric system functions slightly faster ($\approx$5%) than a symmetric transporter (13), but the improvement is unlikely to be sufficient to exert significant selection pressure for asymmetry. Thus, the opposite asymmetries for HCO_3^- and Cl^- may simply be the result of small free-energy differences between the various conformations of human AE1, in which case AE1s from other organisms might exhibit different asymmetry, a prediction that remains to be tested.

Even if the asymmetry of the system is simply caused by the lack of selection pressure to make it symmetric, it is nevertheless somewhat surprising that a system with no external energy input exhibits such pronounced side-dependent asymmetry in substrate affinities and translocation rates. Because of this asymmetry, the conformation of AE1 can be altered from predominately inward-facing to predominately outward-facing simply by substituting HCO_3^- for Cl^- in the medium. This previously uncharacterized asymmetry thus provides a means for experimentally altering the protein conformation to determine what changes in molecular structure occur during the transport process (Ei $\leftrightarrow$ Eo), both in AE1 and possibly other physiologically important coupled-transport systems, if they exhibit similar asymmetry.

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